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Diminished adenylate cyclase activity and aquaporin 2 expression in acute renal failure rats

Soo W. Kim, Youn S. Jeon, Jong Un Lee, Dae G. Kang, Hyun Kook, Kyu Y. Ahn, Sung Z. Kim, Kyung W. Cho, Nam H. Kim, Jin S. Han, and Ki C. Choi

Departments of Internal Medicine, Physiology, Pharmacology, and Anatomy, Chonnam University Medical School, Kwangju; Department of Urology, Sooncheonhyang University Hospital, Chonan; Department of Physiology, Chonbuk National University School of Medicine, Chonju; and Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea

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Background. The present study was aimed at investigating the changes of aquaporin 2 (AQP2) expression and its underlying mechanisms in ischemic acute renal failure (ARF).

Methods. ARF was induced by clamping the both renal arteries for 60 minutes in rats. Two or seven days later, AQP2 expression and trafficking were determined in the kidney by Western blot analysis and immunohistochemistry. The activity of adenylate cyclase was also measured.

Results. The urinary flow rates in ARF-2 and ARF-7 day were significantly increased in association with decreases of urine osmolality. While there was decreased expression of AQP2 in the cortex, outer medulla, and inner medulla in ARF, it was most pronounced in the outer medulla. The AQP2 expression was reduced in the apical membrane-enriched fraction as well the subapical vesicle-enriched fraction in ARF; however, the degree was greater in the former than in the latter. Immunohistochemical study also showed a markedly decreased expression of AQP2 in the collecting duct in ARF. cAMP generation in response to arginine vasopressin (AVP) in the kidney was attenuated in ARF, most prominently in the outer medulla. cAMP generation in the outer medulla in response to forskolin was not affected, but sodium fluoride was significantly blunted in ARF.

Conclusions. The AVP-stimulated adenylate cyclase activity is impaired in ARF, secondary to a defect at the level of the G protein. The expression of AQP2 was reduced as a consequence, which may in part account for urinary concentration defect in ARF.

Acute renal failure (ARF) may result from various pathophysiological states, including sudden decreases of renal blood flow, rapid reduction of glomerular function, and toxic or obstructive injury to the renal tubule [1]. Among other factors, an ischemic insult has been the leading cause of ARF in humans [2]. A 24-hour urine volume of less than 400 mL is compatible with the diagnosis of oliguric ARF in humans [3]. In as many as half of the cases of ARF, however, daily urine volume may exceed this amount, and these patients are diagnosed as having nonoliguric ARF [4]. Patients with nonoliguric ARF appear to have a significant urinary concentration defect [5, 6]. Although the failure to build up a high osmolality in the medullary interstitium may explain the concentration defect in ARF [7, 8], its underlying mechanisms are not yet fully understood.

Arginine vasopressin (AVP) is well known to play a key role in maintaining the body water balance through increasing the water permeability in the collecting duct. The action of binding AVP to the V2 receptor is coupled to the heterotrimeric G protein Gs, which leads to the activation of adenylate cyclase and generation of the hormone's second messenger, cAMP [9]. cAMP may then have a stimulatory role in aquaporin 2 (AQP2) water channel activity in the collecting duct [10].

Short-term regulation of AQP2 water channels exerted by AVP comprises their exocytic insertion into the apical plasma membrane [11–13]. Furthermore, AVP has a long-term effect to increase the abundance of AQP2 proteins in the principal cells of the collecting duct [14]. The expression of AQP2 proteins may be up-regulated in conditions with water retention, such as congestive heart failure [15]. Conversely, it may be down-regulated in a number of acquired nephrogenic diabetes insipidus, such those caused by chronic lithium treatment [16], hypokalemia [17], hypercalcemia [18], and bilateral ureteral obstruction [19]. An impairment of AQP mechanisms may be related to the urinary concentration defect in ARF. Indeed, recent studies using an ischemia-induced ARF rat model have suggested that AQP2 abundance is decreased in the collecting duct [20, 21].

Key words: acute renal failure, cAMP, urinary concentration defect, nonoliguric ARF, AQP2 proteins, ischemia.

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The present study further investigates the mechanisms underlying the altered AQP2 expression in ARF. We determined the AQP2 expression and targeting by Western blot analysis and immunohistochemistry, and measured adenylate cyclase activity in the kidney of rats with ARF induced by an ischemic-reperfusion injury.

METHODS

Experimental animals

Male Sprague-Dawley rats weighing 200 to 250 g were used throughout the study; they were kept in accordance with the Institutional Guidelines of Experimental Animal Care and Use. To induce ARF, both renal arteries were clamped for 60 minutes under ketamine anesthesia (50 mg/kg, intraperitoneally). Control rats underwent sham operation without clamping the renal arteries. They were returned to the cage and were kept two or seven days until used.

Renal function

On the day of experiment, the rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The urinary bladder was exposed through a low midline abdominal incision and cannulated with PE 50 tubing to collect urine samples for one hour. At the end of the urine collection, trunk blood was collected by decapitation. Creatinine clearance (C_{Cr}) and free water reabsorption were measured.

Protein preparation and Western blot analysis

The kidney was rapidly isolated following the decapitation of the animal while under a conscious state and was rapidly frozen. The cortex, outer medulla, and inner medulla from frozen kidney tissues were dissected and were homogenized at 3000 r.p.m. in a solution containing 250 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L phenylmethylsulfonyl fluoride, and 10 mmol/L Tris-HCl buffer, at pH 7.6. Large tissue debris and nuclear fragments were removed by two low-speed spins in succession (1000 $\times$ *g* for 10 min and 10,000 $\times$ *g* for 10 min). Protein samples (cortex, 100 μg; outer medulla, 6 μg; inner medulla, 3 μg) were loaded and electrophoretically size-separated with a discontinuous system consisting of a 12.5% polyacrylamide resolving gel and 5% polyacrylamide stacking gel. The proteins were then electrophoretically transferred to a nitrocellulose membrane at 20 V overnight. The membranes were washed in Tris-based saline buffer (pH 7.4) containing 0.1% Tween-20 (TBST), blocked with 5% nonfat milk in TBST for one hour, and incubated with a 1:750 dilution of antirabbit polyclonal AQP2 antibody (Alomone Lab, Jerusalem, Israel) in 2% nonfat milk/TBST for one hour at room temperature. The membranes were then incubated with a horseradish peroxidase-labeled goat antirabbit IgG (1:1200) in 2% nonfat milk in TBST for two hours. The bound antibody was detected by enhanced chemiluminescence (Amersham, Little Chalfont, Buckinghamshire, UK) on hyperfilm. The relative protein levels were determined by analyzing the signals of autoradiograms using the transmitter scanning videodensitometer.

Differential centrifugation

Differential centrifugation was carried out as previously described [22, 23]. AQP2 trafficking was assessed by comparing the magnitude of its expression in the apical membrane-enriched and subapical vesicle-enriched fractions using differential centrifugation. The homogenate was centrifuged at low-speed spins (1000 $\times$ *g* for 10 min) to remove cell debris and nuclear fragments. The supernatant was further centrifuged at 17,000 $\times$ *g* for 20 minutes to yield apical membrane-enriched pellets. The supernatant was centrifuged again at 100,000 $\times$ *g* for one hour to obtain a vesicle-enriched pellet. Trafficking was noted by the ratio of the high-density fraction (17,000 $\times$ *g*) to the low-density fraction (100,000 $\times$ *g*). A decrease in the HD/LD ratio may then reflect an inhibited trafficking action of AQP2.

Immunohistochemistry

The expression of AQP2 was also determined by immunohistochemistry. Rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally), and the kidneys were fixed by in vivo perfusion of the abdominal aorta with periodate-lysine-paraformaldehyde for 10 minutes. The kidneys were excised and cut into 2 mm coronal slices, which were then immersed in the same fixative overnight at 4°C. The slices were washed in phosphate-buffered saline (PBS), dehydrated in a graded series of ethanol washes, and embedded in paraffin. Tissue sections were made at 2 μm and mounted on gelatin-coated glass slides.

Immunohistochemistry was performed using an immunoperoxidase procedure (Vector Lab, Burlingame, CA, USA) The tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, rinsed two times in PBS, and then treated with 3% H_2O_2 in 60% methanol for 30 minutes to quench endogenous peroxidase activity. After washing twice (5 min each) in PBS, the sections were blocked in PBS containing 5% normal goat serum for one hour. The sections were incubated for 12 to 14 hours with AQP2 antibodies diluted 1:2000 in PBS with 0.3% bovine serum albumin. For a negative control, the sections were incubated in PBS containing 5% normal goat serum only. The sections were then rinsed three times in PBS and incubated for 30 minutes with the biotinylated secondary antibody, followed by a six-minute incubation with the peroxidase substrate solution contained within

Table 1. Changes in functional parameters from ARF-2 and ARF-7 day rats

	ARF-2 day		ARF-7 day	
	Control ($N = 7$)	ARF ($N = 6$)	Control ($N = 7$)	ARF ($N = 6$)
P_{Cr} *mg/dL*	0.40 ± 0.02	1.98 ± 0.11[b]	0.40 ± 0.03	0.52 ± 0.07
C_{Cr} *mL/min*	1.23 ± 0.16	0.39 ± 0.12[c]	1.42 ± 0.15	1.52 ± 0.27
Urinary flow rate *μL/h*	221.2 ± 27.8	737.5 ± 146.1[a]	255.6 ± 29.9	1901.6 ± 397.9[b]
P_{Osm} *mOsm/kg* H_2O	282.6 ± 9.0	340.3 ± 7.0[b]	277.9 ± 12.5	325.2 ± 8.6[a]
U_{Osm} *mOsm/kg* H_2O	1563.5 ± 143.3	594.6 ± 70.1[c]	1688.4 ± 167.4	472.4 ± 53.8[c]
$(U/P)_{Osm}$	5.67 ± 0.6	1.75 ± 0.2[c]	6.2 ± 0.8	1.4 ± 0.1[c]
T^cH_2O *μL/min/kg*	68.9 ± 10.5	35.7 ± 12.0[a]	84.2 ± 9.3	44.4 ± 13.1[a]

Values are mean ± SEM. *N* is the number of experiments. Abbreviations are: P_{Cr}, plasma creatinine; C_{Cr}, creatinine clearance; P_{Osm}, plasma osmolality; U_{Osm}, urinary osmolality; $(U/P)_{Osm}$, urine-to-plasma osmolality ratio; T^cH_2O, solute-free water reabsorption; ARF, acute renal failure.
[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ vs. control

the kit. Finally, the tissue sections were examined and photographed on a light microscope.

Membrane preparation and adenylate cyclase activity

The membrane preparation was obtained as described previously [24]. The renal cortex and outer and inner medulla were separated and homogenized in ice-cold homogenizing buffer (50 mmol/L Tris-HCl, pH 8.0, containing 1 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 250 mmol/L sucrose) and centrifuged at 1000 × *g* and 100,000 × *g* in succession. The resulting pellet was used as membrane preparation. Protein concentrations were measured by bicinchonic acid assay kit (Bio-Rad, Hercules, CA, USA).

Adenylate cyclase activity was assayed by the method of Bar [25], with a slight modification. It was provoked by AVP, sodium fluoride, or forskolin. AVP was used to activate the V2 receptor. Sodium fluoride was used to activate adenylate cyclase in a receptor-independent but guanyl nucleotide-regulatory protein (G-protein)-dependent manner, and forskolin was used to probe the catalytic unit of the adenylate cyclase complex. The reaction was started by adding the membrane fraction (of which the protein contents were 20, 10, and 10 μg for the renal cortex, outer medulla, and inner medulla, respectively) to 100 μL working solution (50 mmol/L Tris-HCl, pH 7.6, containing 1 mmol/L ATP, 20 mmol/L phosphocreatine, 0.2 mg/mL creatine phosphokinase, 6.4 mmol/L $MgCl_2$, 1 mmol/L 3-isobutyl-1-methylxanthine, 0.02 mmol/L GTP). After 15 minutes, the reaction was stopped by cold application of solution consisting of 50 mmol/L sodium acetate, pH 5.0, and centrifuged at 1000 × *g* for 10 minutes at 4°C.

cAMP was measured in the supernatant by equilibrated radioimmunoassay. Iodinated 2′-O-monosuccinyl-adenosine 3′,5′-cyclic monophosphate tyrosyl methyl ester (^{125}I-ScAMP-TME) was prepared as described by previous investigators [26]. Standards or samples were taken up in a final volume of 100 μL of 50 mmol/L sodium acetate buffer (pH 4.8). One hundred microliters of diluted cAMP antiserum (Calbiochem-Novabiochem, San Diego, CA, USA) and ^{125}I-ScAMP-TME (10,000 cpm/100 μL) were then added and incubated for 15 hours at 4°C. The bound form was separated from the free form by charcoal suspension, and the supernatant was counted in a gamma counter (Packard Instrument, Meriden, CT, USA). All samples in one experiment were analyzed in a single assay. Nonspecific binding was <2.0%. The 50% intercept was at 16.5 ± 0.8 fmol/tube ($N = 10$). The intra-assay and interassay coefficients of variation were 5.0 ± 1.2 ($N = 10$) and 9.6 ± 1.9% ($N = 10$), respectively. Results were expressed as moles of cAMP generated per milligram of protein per minute.

Drugs and statistical analysis

Drugs were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless stated otherwise. Results are expressed as mean ± SEM. The statistical significance of differences between the groups was determined using analysis of variance or unpaired *t*-test.

RESULTS

Functional parameters

Table 1 summarizes the functional data in ARF. In ARF-2 day, the plasma creatinine level was significantly increased, along with a decrease of its renal clearance. The urinary flow rate in ARF was significantly increased, in association with decreases in urine osmolality and urine-to-plasma ratio of osmolality. The free water reabsorption (T^cH_2O) was also lower in ARF than in control. In ARF-7 day, renal function showed a partial recovery, in which the plasma creatinine and its renal clearance did not significantly differ between ARF and control rats. However, urinary flow rate was kept increased, along with decreased urine osmolality and urine-to-plasma ratio of osmolality. T^cH_2O was persistently decreased in ARF-7 day rats.

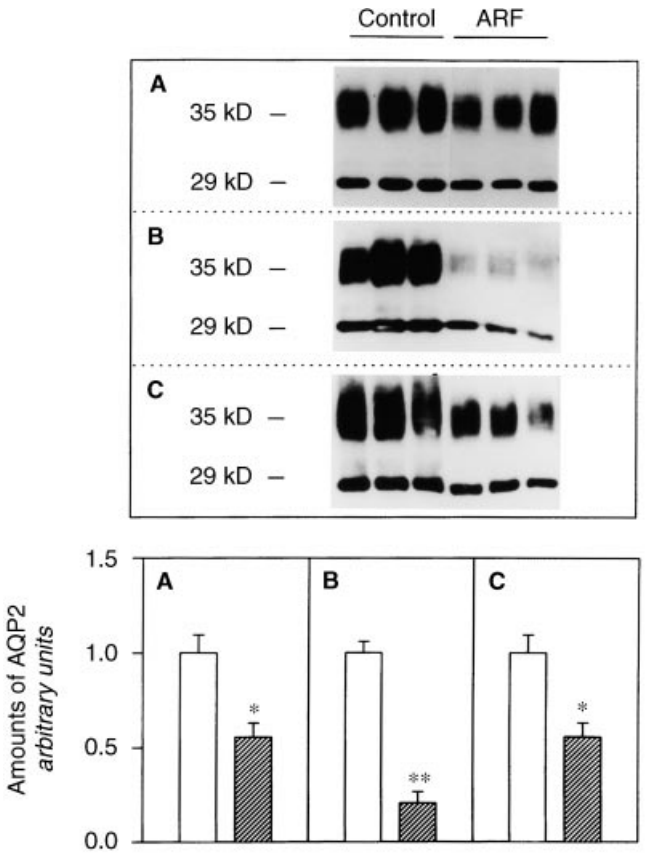

Fig. 1. Western blot analysis of aquaporin 2 (AQP2) in the kidney of control and acute renal failure (ARF)-2 day rats. Immunoblots of AQP2 in the cortex (*A*), outer medulla (*B*), and inner medulla (*C*). Each column represents the densitometric analysis, showing mean ± SEM of six rats. Symbols are: (□) control; (▨) ARF. $*P < 0.05$; $**P < 0.01$ vs. control.

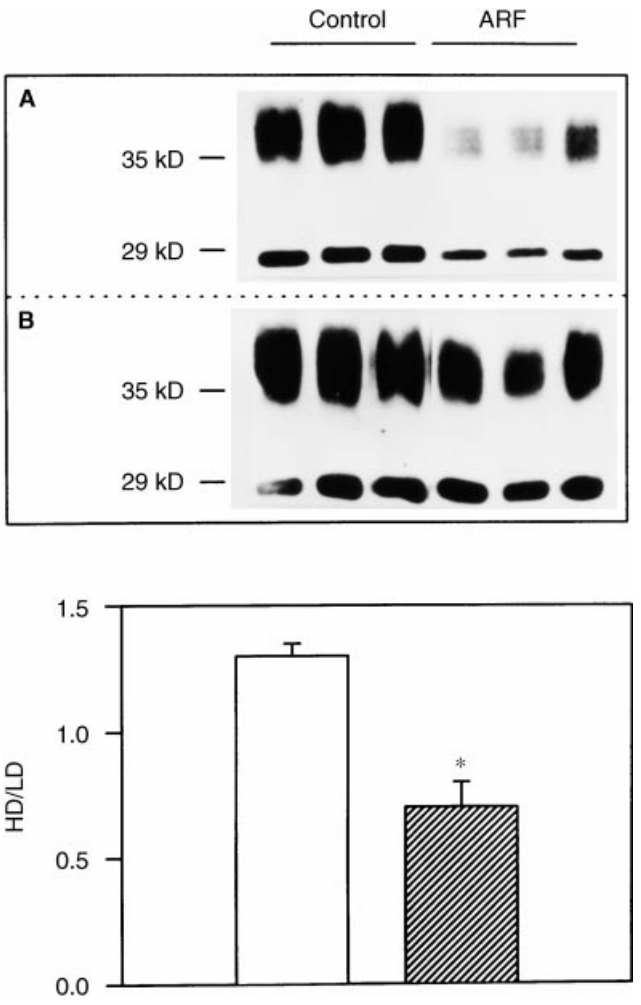

Fig. 2. Differential centrifugation of total kidney extracts in control and ARF-2 day rats. Abundance of AQP2 in the high-density membrane fraction (*A*) and low-density fraction (*B*). The immunoblot was reacted with affinity-purified anti-AQP2. Each column represents the ratio of the high-density membrane fraction over low-density fraction (HD/LD), showing mean ± SEM of eight rats. $*P < 0.05$ vs. control.

Expression of aquaporin 2 proteins

The expression of AQP2 proteins was determined in the kidney cortex, outer medulla, and inner medulla. The anti-AQP2 antibody recognizes 29 kD and 35 to 50 kD bands, corresponding to nonglycosylated and glycosylated AQP2, respectively. A significant decrease of AQP2 expression was noted in the cortex (Fig. 1A), outer medulla (Fig. 1B), and inner medulla (Fig. 1C) in ARF-2 day, being most pronounced in the outer medulla. Differential centrifugation techniques were employed to study regulated trafficking of AQP2. The AQP2 expression was decreased in the apical membrane-enriched fraction, as well as in the subapical vesicle-enriched fraction. Furthermore, the ratio of HD/LD was decreased in ARF (Fig. 2).

Figure 3 shows AQP2 protein expression in the kidney of ARF-7 day rats. The expression was significantly decreased in the cortex (Fig. 3A), outer medulla (Fig. 3B), and inner medulla (Fig. 3C), the degree of which was more pronounced in the medulla than in the cortex. In addition, the ratio of HD/LD was decreased in ARF (Fig. 4).

Immunohistochemistry

Immunohistochemistry was carried out using 2 μm sections of the kidney. The immunohistochemistry showed an abundance of AQP2 labeling exclusively in the principal cells of collecting duct, being most prominent in the inner medulla. It was distributed both in the apical region of the cell and throughout the cytoplasm. In ARF, there was a marked decrease in the AQP2 labeling, the degree of which was most prominent in the outer medulla. The residual AQP2 was located mainly in the cytoplasmic region of the principal cell (Fig. 5).

Adenylate cyclase activity

Figure 6 shows cAMP generation in response to graded doses of AVP in the cortex, outer medulla, and inner medulla. The cAMP generation was blunted in

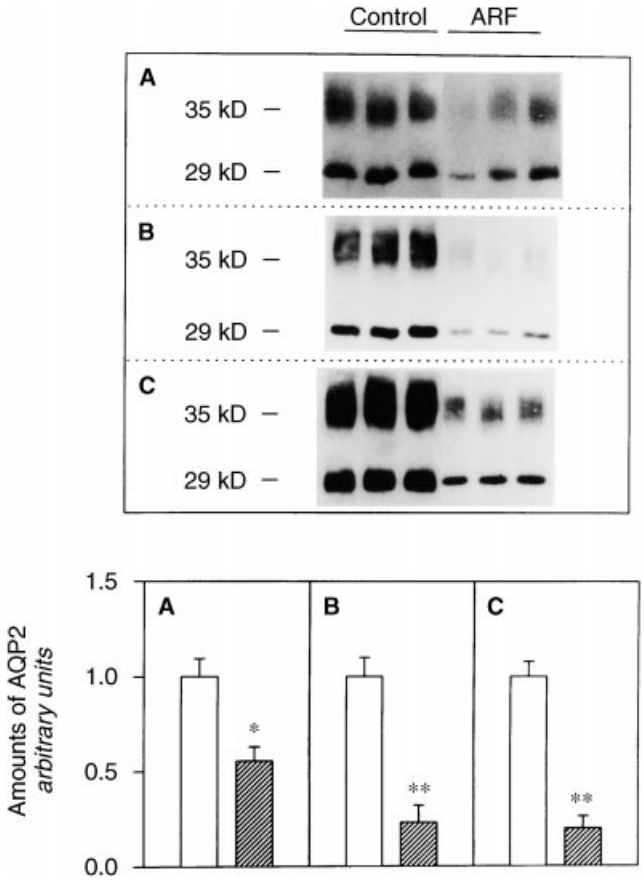

Fig. 3. Western blot analysis of AQP2 in the kidney of control and ARF-7 day rats. Immunoblots of AQP2 in the cortex (*A*), outer medulla (*B*), and inner medulla (*C*). Each column represents the densitometric analysis, showing mean ± SEM of six rats. Symbols are: (□) control; (▨) ARF. $*P < 0.05$; $**P < 0.01$ vs. control.

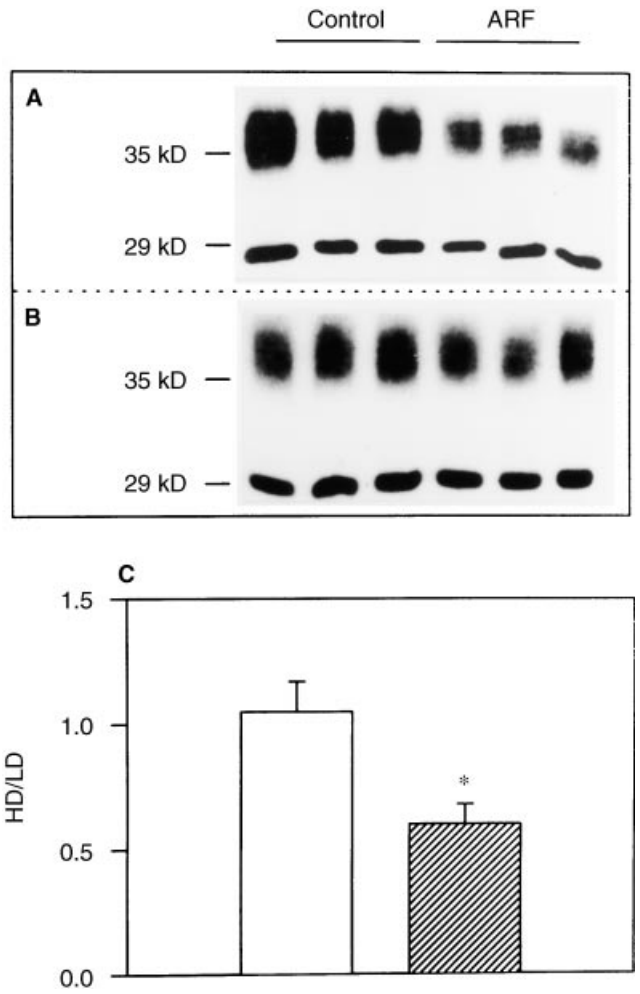

Fig. 4. Differential centrifugation of total kidney extracts in control and ARF-7 day rats. Abundance of AQP2 in the high-density (HD) membrane fraction (*A*) and low-density (LD) fraction (*B*). Each column represents the ratio of HD/LD, showing mean ± SEM of eight rats. $*P < 0.05$ vs. control.

ARF, the degree of which was most prominent in the outer medulla. Therefore, adenylate cyclase activity was further studied with sodium fluoride and forskolin in the outer medulla. The cAMP generation stimulated by forskolin did not differ between the ARF and control groups, but the sodium fluoride response was significantly impaired in the groups with ARF (Fig. 7).

DISCUSSION

Acute renal failure was induced by bilateral ischemia and reperfusion injury in the present study. At two days after the ischemia-reperfusion injury, the plasma creatinine level was significantly increased along with a decrease of its renal clearance, thus showing uremic renal failure. However, ARF-7 day showed a partial recovery from the renal failure, as noted by a normalization of the plasma level and renal creatinine clearance. The urine-to-plasma ratio of osmolality and tubular free water reabsorption was also decreased, indicating an impaired urinary concentrating ability. Accordingly, urinary flow rates were markedly increased, with the degree being more prominent in ARF-7 day than in ARF-2 day. The decreased glomerular filtration rate in ARF-2 day may have limited further increases of urinary excretion despite the reduced concentrating ability, so that the polyuria was less marked.

The actions of AVP are mediated by two main subtypes of membrane-bound receptors [27]. In the kidney, the V2 receptor is coupled to adenylate cyclase, which consists of three major parts: receptor, G protein, and catalytic unit [28]. G protein acts as a transducer and sends a signal from the hormone-occupied receptor to the catalytic unit [9, 29]. The catalytic unit then induces the final enzymatic activity responsible for ATP hydrolysis and cAMP generation. The generated cAMP in turn has a stimulatory role in short- and long-term regulation of AQP2 [11, 30]. Short-term regulation represents a cAMP-dependent insertion of AQP2-containing vesicles to the

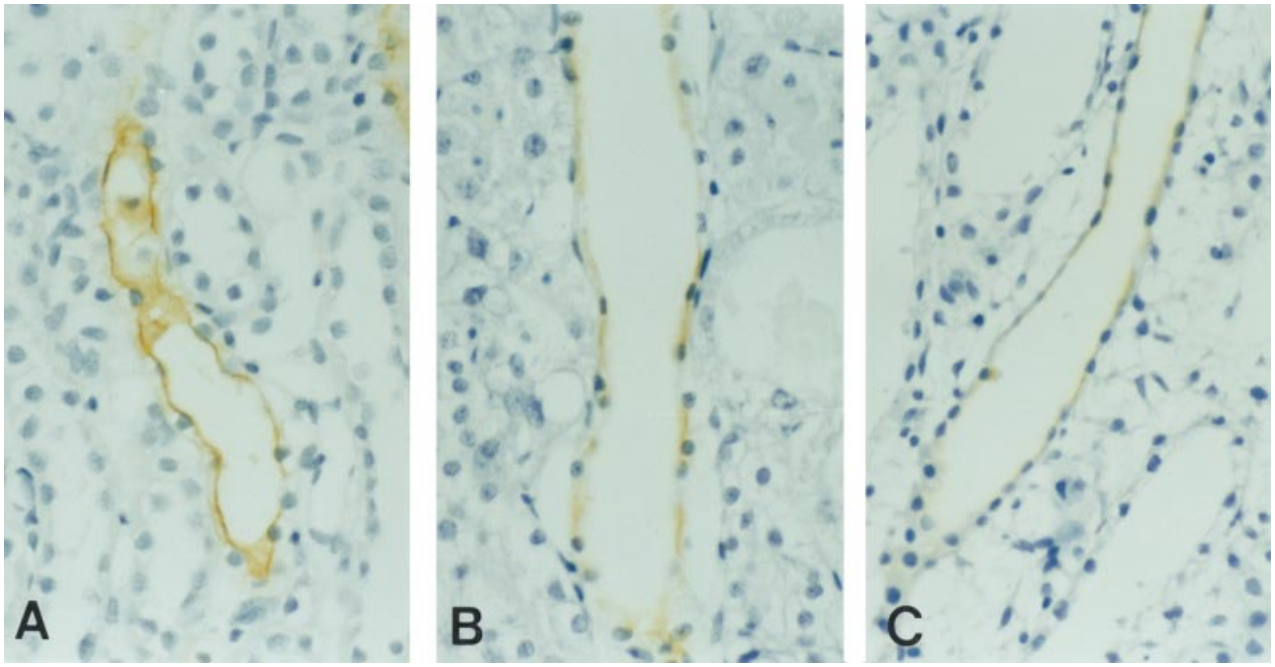

Fig. 5. Immunohistochemical localization of AQP2 in the outer medulla in control (*A*), ARF-2 day (*B*), and ARF-7 day (*C*) rats. AQP2 labeling is found in both the apical plasma membrane and cytoplasmic domains. In ARF, there was a marked decrease in AQP2 labeling. Magnification ×360. Publication of this figure in color was made possible by a grant from MSD Korea Ltd, Kwangju, Korea.

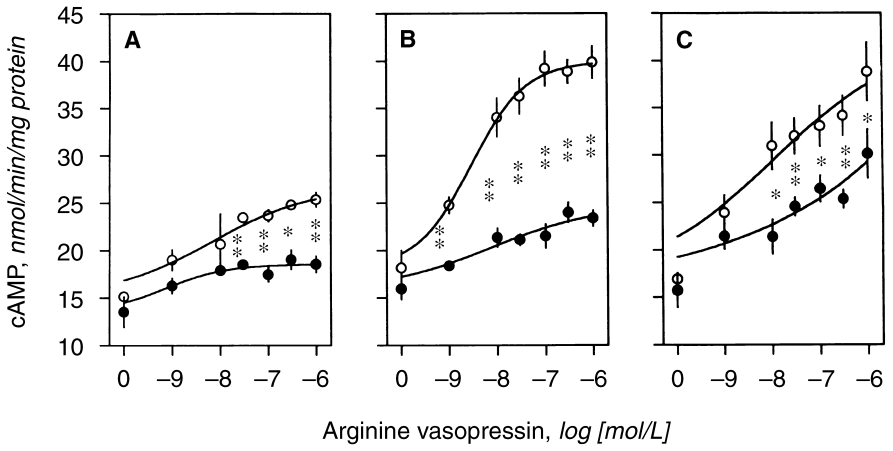

Fig. 6. cAMP production in response to arginine vasopressin (AVP) in the cortex (*A*), outer medulla (*B*), and inner medulla (*C*) in control and ARF-2 day rats. Symbols are: (○) control rats; (●) ARF-2 day rats. Each point represents the mean ± SEM of six experiments. $*P < 0.05$; $**P < 0.01$ vs. control.

apical membrane, which is rapidly reversible upon dissociation of AVP from its receptor [11]. Therefore, the rapid increase in water permeability seen with AVP stimulation may be attributed to a redistribution of AQP2-containing membrane domains within the collecting duct cell to increase the number of AQP2 water channels in the apical membrane at the expense of intracellular AQP2 vesicles [11–13].

In the present study, ARF was associated with an altered regulation of AQP2 water channels in the kidney. Two modes of dysregulation were suggested: a decrease in the expression of AQP2 protein, and an impairment of AQP2 trafficking in the collecting duct. The AQP2 expression was significantly reduced in the apical membrane-enriched, as well as in the subapical vesicle-enriched fraction in ARF; however, the degree of expression was greater in the former. Immunohistochemical study also showed that there was a marked decrease in AQP2 labeling in the collecting duct. Although the residual AQP2 was located mainly in the cytoplasmic region of the principal cell, it was still observed in the apical plasma membrane. This finding may be in line with a recent study reported by Kwon et al [20]. They demonstrated that AQP2 and AQP3 levels were de-

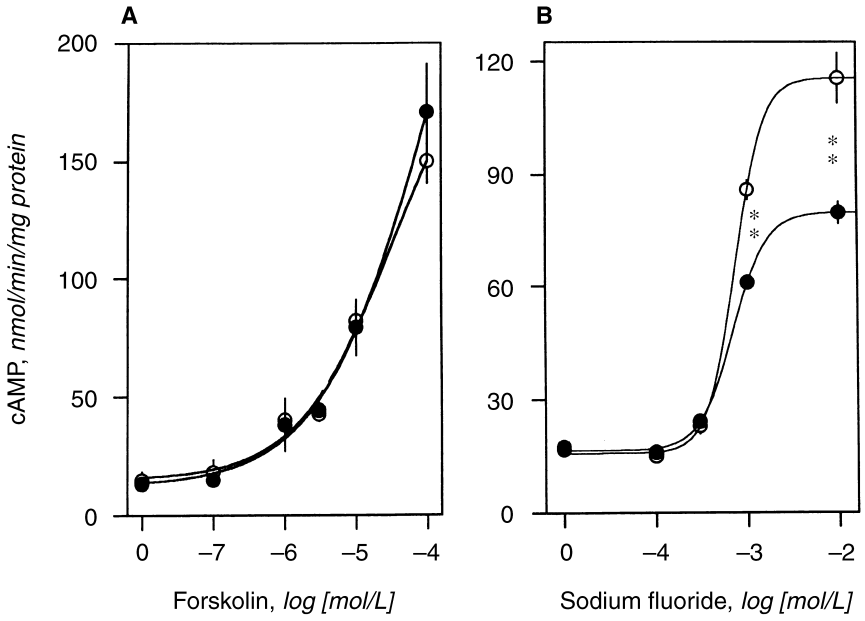

Fig. 7. cAMP production in response to forskolin and sodium fluoride in the outer medulla in the control and ARF-2 day rats. Symbols are: (○) control rats; (●) ARF-2 day rats. Each point represents mean ± SEM of five experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control.

creased in the collecting duct after bilateral ischemic insult, and part of the remaining AQP2 labeling was associated with apical plasma membrane domains. These findings suggest that AQP2 trafficking is not severely impaired in ARF. Fernandez-Llama et al also demonstrated the reduction in AQP2 levels with its maintained targeting in ARF induced by unilateral renal ischemia of 45 minutes with contralateral nephrectomy [21]. The degree of the impaired trafficking may reflect the differences in ischemia duration to induce ARF or animal models.

Long-term regulation exerted by AVP, on the other hand, increases the expression of AQP2 proteins [14]. AQP2 transcription is mediated by cAMP-dependent phosphorylation and subsequent binding of cAMP response element (CRE)-binding protein to the CRE in the promoter region of the AQP2 gene [30]. Therefore, a prolonged impairment in cAMP generation leads to decreases of AQP2 expression and hence of water reabsorption in the kidney. We observed that cAMP generation in response to AVP was blunted in ARF. Furthermore, the cAMP generation was blunted more drastically in the outer medulla, as was the decrease of AQP2 expression, than in the cortex or inner medulla. The tubular segments located within the outer medulla have been suggested to suffer the most severe injury after an ischemic insult because of their high ATP requirement for active solute transport and the regional difference in renal blood flow that renders the outer medulla more hypoxic than other regions of the kidney [31]. Taken together, in ARF, a long-term dysregulation of AQP2 occurs as a consequence of an impaired cAMP generation, especially in the outer medulla.

To further specify the point of impaired cAMP generation, we separately examined the adenylate cyclase complex. In response to forskolin, which directly activates the catalytic unit of adenylate cyclase [32], no impairment in cAMP generation was observed in ARF. However, adenylate cyclase activity in response to sodium fluoride, which activates adenylate cyclase in a receptor-independent but G-protein–dependent manner [33, 34], was blunted in ARF. These findings indicate that the catalytic unit of the adenylate cyclase complex may not be affected in ARF, but G protein may be the site of derangement among the cAMP generation cascades.

The medullary osmotic gradient determining the degree of water permeability and urinary concentration in the collecting duct is primarily produced by the thick ascending limb of Henle's loop (TAL), being known as a countercurrent multiplier mechanism [35]. Salt reabsorption in TAL involves the bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransport in the apical membrane, in which AVP may also have a regulatory role [36]. Therefore, the attenuated cAMP generation in response to AVP in the outer medulla may decrease Na^+-K^+-$2Cl^-$ cotransport, also contributing to the urinary concentration defect in ARF. This possibility remains to be further elucidated.

In summary, our results suggest that the urinary concentration defect and increased water excretion in ARF is, at least in part, accounted for by a reduced abundance of AQP2 water channels in the collecting duct. However, the primary impairment may lie at the level of membrane G protein that acts as a transducer between AVP receptor and adenylate cyclase, decreasing the AVP-stimulated adenylate cyclase activity and hence the subsequent generation of cAMP.

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Reprint requests to Ki Chul Choi, M.D., Department of Internal Medicine, Chonnam University Medical School, 8 Hak-dong, Kwangju 501-757, Korea.
E-mail: choikc@chonnam.chonnam.ac.kr

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